Switching Binding Sites: Low-Temperature NMR Studies on Adenosine−Aspartic Acid Interactions

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Low-temperature NMR experiments were performed on mixtures of adenine nucleosides and aspartic acid derivatives in a freonic solvent. By acquiring spectra at temperatures as low as 123 K, the regime of slow hydrogen bond exchange is reached and hydrogen-bonded complexes can be characterized in detail. With 2′-deoxyadenosine lacking a 2′-substituent, *N*-Boc-protected aspartic acid benzyl ester binds through its carboxylic acid side chain to the Watson−Crick site of the adenine base, forming a strong hydrogen bond with the proton located close to the center between the oxygen donor and adenine N1 nitrogen acceptor. However, in the case of 2′-*O*-silylated adenine ribofuranosides, noncovalent interactions of the 2′-substituent with protecting groups on the amino acid shift the binding mode toward a Hoogsteen geometry with only a moderately strong hydrogen bond involving adenine N7.

Introduction

The detailed characterization of hydrogen bond interactions between biomolecular building blocks has been the subject of numerous experimental and theoretical studies in the past. Besides gas-phase spectroscopy on isolated molecules and clusters,[1] NMR spectroscopy has been established as one of the most powerful tools to study the strength and geometry of hydrogen bonds in both the solid and the liquid state.[2] For the latter, however, NMR signals at ambient temperatures generally correspond to an average over fast exchanging hydrogen-bonded species, thus restricting a detailed characterization of hydrogen bonds for individual complexes. To circumvent problems arising from fast exchange in weakly hydrogen-bonded systems, measurements have to be performed at very low temperatures where the regime of slow hydrogen bond exchange within the NMR time scale is reached. In the liquid state, significant progress has recently been achieved by employing deuterated freonic mixtures as NMR solvents. Such solvents allow high-resolution NMR measurements down to 100 K and enable a detailed characterization of even weakly hydrogen bonded associates in the slow exchange regime.[3] Also, the temperature-dependent dielectric constant of the Freon solvent of up to 40 at very low temperatures[4] may closely mimic the relatively apolar microenvironment in many binding domains,[5,6] which determines the strength of hydrogen bond interactions within biomolecular complexes.

Hydrogen bond interactions between adenine and other nucleobases are critical for biological recognition events that determine the genetic code as well as nucleic acid secondary and tertiary structural elements. In addition, the hydrogen bond mediated specific recognition of adenine by amino acid residues plays a vital role in the binding of transcription factors to DNA or to the binding of adenine cofactors to their binding sites in enzymes. Many hydrogen bonds between amino acids and nucleobases are involved in bidentate interactions that provide for a higher specificity. Thus, in protein−DNA interactions bidentate hydrogen bonding is frequently observed between asparagine or glutamine side chains and the adenine base.[7] Although generally deprotonated under physiological conditions, the protonated amino acid residues of aspartic and glutamic acid may likewise play an important role in the hydrogen bond mediated recognition of adenine within hydrophobic binding pockets that are protected from the aqueous environment. We have recently reported on the binding of unsubstituted and chloro-substituted acetic acid to the adenine base.[8] These studies demonstrated that the Watson−Crick face of adenine is the preferred binding site for these simple carboxylic acids forming stronger OH···N hydrogen bonds to adenine N1 when compared to N7 at the Hoogsteen face. Also, increasing the acidity and thus the proton donating power of the carboxylic acid will gradually shift the proton toward the adenine nitrogen acceptor within the complex ultimately forming ion pairing species. We now report on the association between an adenine nucleoside and an N- and C-protected aspartic acid. Due to its various functionalities, the amino acid will be shown to add more complexity to its adenine binding and additional noncovalent interactions must be taken into account for a full understanding of the complex formation.

Experimental Methods

Materials. Reagents of the highest quality available were purchased from Sigma-Aldrich, Deisenhofen, Germany. Boc-L-Asp-OBzl (Boc = *tert*-butyloxycarbonyl and OBzl = benzyl ester) and Ac-L-Asp-OMe were obtained from Bachem, Germany, and used without further purification after NMR analysis showed the absence of impurities. $^{15}NH_4Cl$ was purchased either from Chemotrade, Leipzig, Germany (95% of label) or Deutero GmbH, Kastellaun, Germany (99% of label). All reactions were controlled by thin-layer chromatography (TLC) on silica gel

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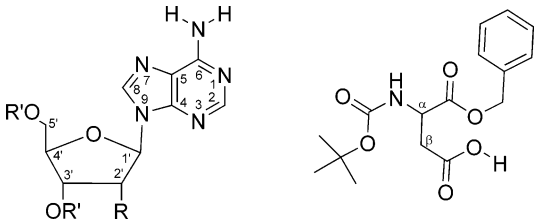

Figure 1. Structure with atom designations of the adenine nucleoside (left) and protected L-aspartic acid Boc-Asp-OBzl (right): R′ = triisopropylsilyl (TIPS) or *tert*-butyldimetylsilyl (TBDMS); R = H or OR′.

plates (Merck silica gel 60 F_{254}). If necessary, solvents were dried by standard procedures prior to use. The deuterated Freon mixture $CDClF_2/CDF_3$ was prepared as described[9] and handled on a vacuum line which was also used for the sample preparation. 3′,5′-Di-*O*-silylated 7-^{15}N-2′-deoxyadenosine and 2′,3′,5′-tri-*O*-silylated 1-^{15}N-adenosine were synthesized as described.[8,10−12]

*[8-*D*]-2′-Deoxyadenosine.* 2′-Deoxyadenosine (2 g, 8 mmol) was dissolved in D_2O (30 mL) and heated to 100 °C. After 6 h H/D exchange was found to be complete by NMR.

NMR Spectroscopy. NMR experiments were performed on a Bruker AMX500 spectrometer. Temperatures were adjusted by a Eurotherm variable temperature unit to an accuracy of ±1.0 °C. Temperature calibration was performed with a sample of methanol in MeOH-d_4 and the calibration curve extrapolated for temperatures outside the range covered by the methanol sample. ^{1}H chemical shifts in the Freon mixture were referenced relative to $CHClF_2$ ($\delta_H = 7.13$ ppm). *J* values are given in hertz. For a typical phase-sensitive 2D nuclear Overhauser effect (NOE) experiment at low temperatures, the spectrum was recorded with a mixing time of 60 or 80 ms and a pulse-recycle time of 1 s. A total of 1*K* free induction decays (FIDs) of 2*K* complex data points were collected using the time proportional phase incrementation (TPPI) method, and the t_1 and t_2 FIDs zero-filled to give a final matrix of $2K \times 1K$ real data points prior to Fourier transformation.

Results and Discussion

To allow for a better solubility in the freonic solvent and to block additional hydrogen bond donor and acceptor sites, the sugar hydroxyl groups of the adenosine nucleoside were either triisopropylsilyl (TIPS) or *tert*-butyldimethylsilyl (TBDMS) protected. Likewise, the backbone amino and carboxyl functional groups of L-aspartic acid were derivatized through Boc and OBzl protecting groups (Figure 1).

Clearly, the protected L-aspartic acid Boc-Asp-OBzl has not only significant conformational degrees of freedom but also several potential hydrogen bond donor and acceptor sites through its carboxylic acid side chain and its backbone amide and ester functionalities. The ability of forming various homoassociates is evident from the low-temperature spectra of a freonic Boc-Asp-OBzl solution exhibiting several hydrogen-bonded COOH protons in slow exchange between 12 and 16 ppm (not shown). Likewise, upon lowering the temperature of a 1:1 mixture of Boc-Asp-OBzl and specifically labeled 3′,5′-di(triisopropylsilyl)−7-^{15}N-2′-deoxyadenosine (7-^{15}N-dA) below 173 K in Freon, various carboxylic acid OH proton signals of the aspartic acid side chain appear at low field in slow exchange. However, as shown in Figure 2a, the high-intensity most deshielded OH proton with a chemical shift of 19.95 ppm points to the formation of a predominant adenosine−aspartic acid complex at 123 K.

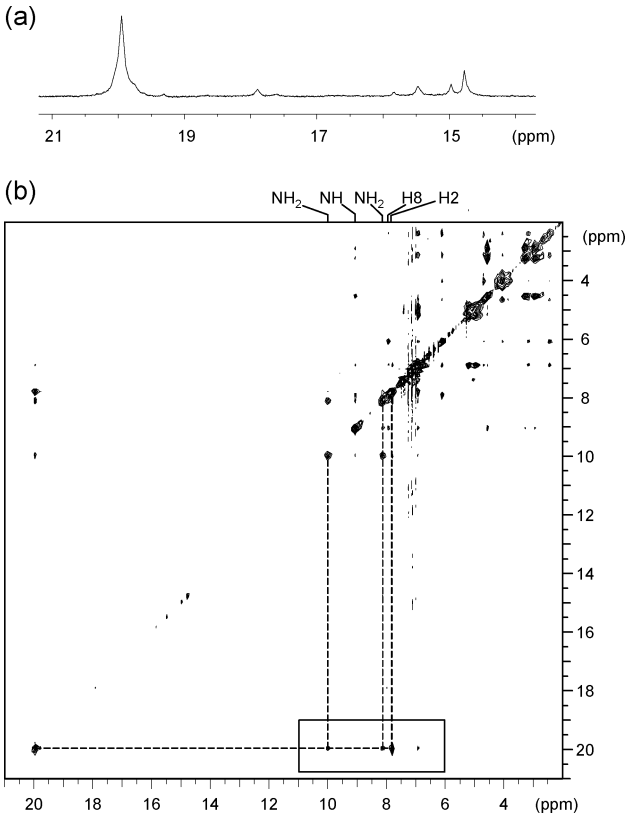

Figure 2. (a) Low-field ^{1}H NMR spectral region and (b) 2D NOE spectrum acquired with an 80 ms mixing time for a 1:1 mixture of 3′,5′-di(triisopropylsilyl)-7-^{15}N-2′-deoxyadenosine and Boc-Asp-OBzl in Freon at 123 K. NOE connectivities between COOH and adenine protons are indicated.

Due to their low signal intensity resulting in the lack of noticeable NOE cross-peaks, we did not attempt to structurally characterize minor associates but rather focused on the unambiguous assignment of the predominant complex formed between the adenine base and the amino acid. As shown in Figure 2b, a 2D NOE spectrum acquired on the 2′-deoxyadenosine−aspartic acid mixture at 123 K shows cross-peaks between the major carboxylic acid OH resonance at 19.95 ppm and the two amino protons of adenosine at 9.99 and 8.14 ppm as well as to another proton at 7.81 ppm. Assuming an anti-glycosidic torsion angle for the nucleoside, the assignment of the latter to H2 of adenine is indicated by the absence of any NOE contact between this resonance and deoxyadenosine sugar protons.[13] However, due to considerable signal broadening and partial overlap of H2 and H8 resonances at very low temperatures, we employed adenosine specifically deuterated in 8-position to exclude any contacts to adenine H8 for an additional independent confirmation of this critical assignment. As shown in Figure 3a for a one-dimensional spectrum acquired at 253 K, deuterium exchange completely eliminated the H8 signal at 8.3 ppm without affecting the H2 resonance at 8.1 ppm in the specifically deuterated sample. Consequently, with a corresponding NOE cross-peak still observed for the 8-deuterated adenine (Figure 3b), the initial proton assignment to H2 could be unambiguously confirmed. Note that the H2 signal shows a gradual upfield shift upon lowering the temperature and finally appears at a chemical shift of 7.81 ppm at 123 K. Another weak NOE connectivity found for the carboxylic acid proton to a resonance at 6.94 ppm is probably due to phenyl protons of the aspartic acid C-protecting group.[14]

Obviously, the aspartic acid preferentially binds to the Watson−Crick side of the adenine base forming a strong

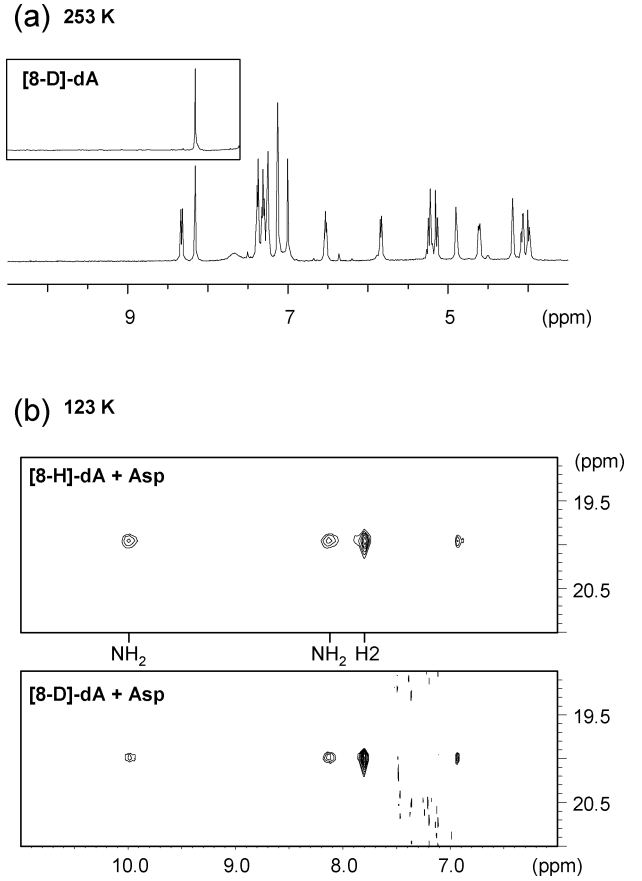

Figure 3. (a) ^{1}H NMR spectrum of a mixture of nondeuterated 3′,5′-di(triisopropylsilyl)-7-^{15}N-2′-deoxyadenosine (full spectrum) and 8-deuterated 3′,5′-di(triisopropylsilyl)-2′-deoxyadenosine (inset) with Boc-Asp-OBzl in Freon at 253 K. (b) 2D NOE spectral region as marked in Figure 2b showing COOH − base proton contacts using nondeuterated (top) and 8-deuterated (bottom) 3′,5′-di(triisopropylsilyl)-2′-deoxyadenosine. The spectra were acquired at 123 K with an 80 ms mixing time.

hydrogen bond to adenine N1. In general, the geometry of hydrogen bonds can be assessed by NMR spectral parameters like ^{1}H NMR chemical shifts or ^{1}H−^{15}N scalar couplings of the proton in the hydrogen bridge. With increasing donating power of the acidic donor, the proton is gradually shifted from O-donor to N-acceptor giving rise to a maximum in the chemical shift at about 21 ppm for a centralized proton and upfield shifts for moving the proton back toward the donor or further toward the acceptor atom.[15] Also, proton transfer from oxygen to nitrogen is conveniently followed by a continuous increase in the J_{NH} scalar coupling with a limiting value of about 90 Hz observed for a complete transfer to form a covalent N−H bond with bond order one. The significantly deshielded acid OH proton with its chemical shift close to 20 ppm indicates its considerable displacement toward the N1 acceptor atom in a central position between the two heavy atoms. Note that the strongly deshielded acid OH proton exhibits no ^{1}H−^{15}N scalar coupling to the specifically 7-^{15}N labeled 2′-deoxyadenosine as would be expected in case of N7 acting as hydrogen bond acceptor.

In an attempt to measure an anticipated trans-hydrogen bond scalar coupling to N1 of adenine, we synthesized a specifically 1-^{15}N-labeled adenine ribofuranoside starting from 6-chloroadenosine. The downfield region of the corresponding ^{1}H NMR spectrum of TBDMS-protected 1-^{15}N-adenosine (1-^{15}N-rA) and the protected aspartic acid in Freon at 123 K is shown in Figure 4a.

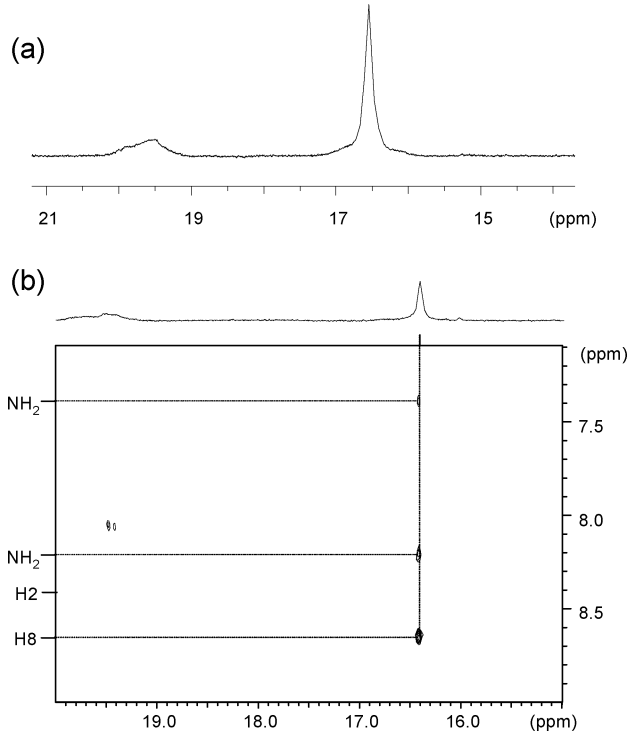

Figure 4. (a) Low-field region of a ^{1}H NMR spectrum at 123 K and (b) portion of a 2D NOE spectrum acquired with an 80 ms mixing time at 133 K for a mixture of 2′,3′,5′-tri(*tert*-butyldimethylsilyl)-1-^{15}N-adenosine with Boc-Asp-OBzl in Freon. NOE connectivities between COOH and adenine protons are indicated.

Unexpectedly, the proton spectrum exhibits a predominant singlet resonance with a chemical shift at 16.55 ppm and thus at much higher field when compared to the mixture with silylated 7-^{15}N-2′-deoxyadenosine. Also, only a rather broad and featureless signal is found in the expected low-field region between 19 and 20 ppm.

To characterize the association mode of 1-^{15}N-rA with aspartic acid, a 2D NOE experiment on the mixture was performed at 133 K. A portion of the spectrum is shown in Figure 4b. For the ribonucleoside, the predominant carboxylic acid OH side chain resonance at 16.42 ppm exhibits NOE connectivities to adenine amino protons at 8.20 and 7.38 ppm as well as to H8 at 8.64 ppm at 133 K. Note the rather upfield-shifted amino protons for this complex whose (de)shielding is a sensitive measure for the extent of adenine N1 protonation that withdraws electrons from the pyrimidine ring system.

A major resolved H2 resonance with a chemical shift of 8.41 ppm is easily identified by its scalar coupling of 14.5 Hz to 1-^{15}N in the specifically labeled adenosine sample.[16] Unlike H8 and as expected for an anti-geometry, it does not exhibit NOE contacts to sugar protons. Obviously, in contrast to its binding to the 2′-deoxyadenosine receptor the aspartic acid binds through its carboxylic acid side chain to the Hoogsteen face of the adenine ribonucleoside forming a hydrogen bond of only moderate strength to adenine N7. The weak NOE connectivity of the broad and low-intensity downfield-shifted carboxylic acid OH signal at about 19.45 ppm (possible ^{1}H−^{15}N scalar couplings are not resolved) is probably due to a small fraction of Watson−Crick bound carboxylic acid exhibiting an NOE contact to a corresponding minor adenine H2 proton. The structures of both predominant complexes as determined from the NOE experiments are shown in Figure 5.

Factors Affecting Aspartic Acid Binding. With Boc-Asp-OBzl binding in different geometries to the adenine nucleobase,

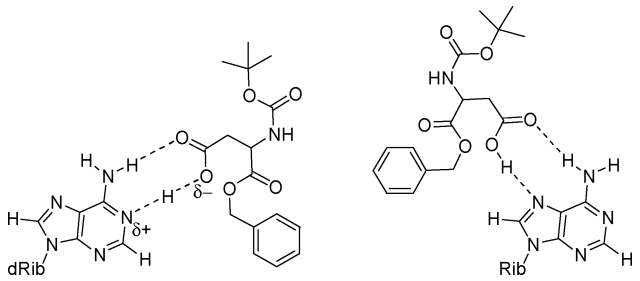

Figure 5. Structure of complexes formed between Boc-Asp-OBzl and 3′,5′-di(triisopropylsilyl)-2′-deoxyadenosine (left) as well as 2′,3′,5′-tri(*tert*-butyldimethylsilyl)-adenosine (right).

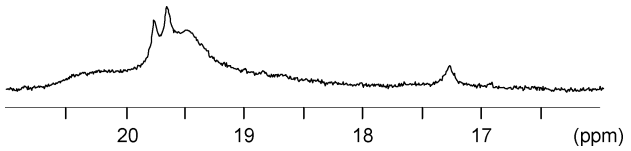

Figure 6. ^{1}H NMR spectrum of 2′,3′,5′-tri(*tert*-butyldimethylsilyl)-1-^{15}N-adenosine and Ac-Asp-OMe (1:1.1 molar ratio) in Freon at 128 K.

other noncovalent interactions must determine the binding preference in the presence of different sugar moieties. Exchanging TIPS and TBDMS protecting groups in the ribo- and 2′-deoxyribofuranoside did not affect the aspartic acid binding mode. Also, 2D NOE cross-peaks between adenine H8 and sugar protons establish an anti-glycosidic torsion angle for both deoxyribose and ribose nucleosides (vide supra). Assuming a fast conformational equilibrium described by N-type (C3′-endo) and S-type (C2′-endo) conformers based on a two-state model, a detailed analysis of ^{1}H−^{1}H scalar coupling constants determined from 1D ^{1}H NMR spectra by employing an optimized Karplus equation parametrized for ribo- and deoxyribonucleosides[17] yields a mole fraction of S-conformer at 273 K of about 0.7 and 0.6 for adenosine and 2′-deoxyadenosine, respectively. According to the observed scalar couplings these populations do not change significantly with temperature or identity of the protecting group and thus exclude any major impact of nucleoside conformation on the different complex geometries.[18]

Consequently, the 2′ sugar substituent in the adenine ribofuranoside is expected to be engaged in additional interactions with the aspartic acid. Indeed, by using only partially silylated 3′,5′-di(*tert*-butyldimethylsilyl)adenosine with a free 2′-OH hydroxyl group, a Watson−Crick geometry with a strongly deshielded OH proton at about 20 ppm is again observed in the major complex (not shown). Likewise, by substituting the bulky *N*-Boc and *O*-benzyl protecting groups of Boc-Asp-OBzl for an *N*-acetylated methyl ester Ac-Asp-OMe, the ^{1}H spectrum acquired at 128 K for a freonic mixture with 1-^{15}N-rA exhibits a major, strongly deshielded COOH doublet at 19.7 ppm in addition to very broad and featureless high-field- and low-field-shifted signals (Figure 6).

Forming a Watson−Crick hydrogen bond as evidenced from its NOE contact to adenine H2, the measured coupling for this proton of $J_{NH} \sim 50$ Hz arises from the trans-hydrogen bond scalar coupling to ^{15}N-labeled adenine N1. With more than half the value found for a single covalent NH bond, this coupling again indicates a considerable displacement of the proton toward the nitrogen acceptor in the hydrogen bridge.

To get a better understanding of possible interactions, we did a Monte Carlo conformational search with the MMFF force field for Hoogsteen and Watson−Crick complexes of Boc-Asp-OBzl and rA while restraining the corresponding hydrogen bonds.

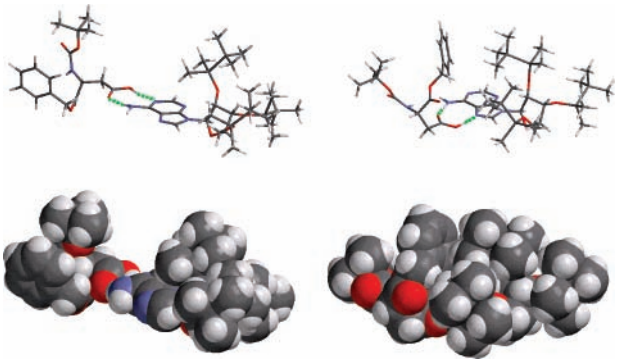

Figure 7. Tube and space-filling models of Monte Carlo optimized structures (2000 steps) of Watson−Crick (left) and Hoogsteen (right) complexes between 2′,3′,5′-tri(*tert*-butyldimethylsilyl)adenosine and Boc-Asp-OBzl.

Characteristic low-energy conformers of the search are depicted in Figure 7 as tube and space filling models.

Whereas in a Watson−Crick geometry substituents of the aspartic acid and adenosine are far removed and lack any contact, Hoogsteen geometries fold into rather compact structures. More specifically, the 2′-*tert*-butyldimethylsilyl protecting group of the ribofuranoside approaches the phenyl ring of the aspartate *C*-benzyl substituent from the top, giving rise to additional van der Waals interactions with increased stabilization compared to the more open Watson−Crick structure. Although calculations have only been performed in vacuo and solvent effects might be significant, the structures of the conformers are expected to nevertheless give an idea about potential noncovalent interactions that are responsible for guiding the binding of the ligand and for overriding anticipated hydrogen bond preferences. It should also be mentioned that torsion angles of the conformers are in excellent agreement with the scalar couplings that have been experimentally determined at higher temperatures.[18]

Higher Order Complexes. In the binding of simple carboxylic acids to the adenine base, ternary or even higher order complexes with both Watson−Crick and Hoogsteen binding sites occupied were often found to form.[8] Thus, with the acid preferentially occupying the Watson−Crick site, a second acid or a second adenine base simultaneously binds to the Hoogsteen site with concomitant weakening or strengthening of the Watson−Crick hydrogen bond. In fact, occupation of both binding sites in adenine is also indicated for the aspartic acid binding. NOE cross-peaks observed between the amidic NH of Boc-Asp-OBzl and adenine amino protons as well as to H8 of bis-silylated 2′-deoxyadenosine or to H2 of tris-silylated adenosine suggest that free Hoogsteen or Watson−Crick faces must be at least partially occupied by a second Boc-Asp-OBzl. As for the carboxylic acid proton, the Watson−Crick bound NH exhibiting an NOE contact to adenine H2 is significantly more deshielded compared to the Hoogsteen bound amide in line with a larger shift of the proton toward the adenine N1 acceptor in a stronger hydrogen bond.[14,16] Interestingly, it is the less acidic amide functionality that is preferred over a second side chain carboxylic acid in forming another cyclic hydrogen bond to adenine, probably exerting a cooperative effect with strengthening of the initially formed hydrogen bonds. Likewise, a second adenine base may also bind to available donor and acceptor sites of the central adenine as has been demonstrated in acetic acid binding at low temperatures previously.[8] Due to different additional higher order complexes that may coexist in solution, corresponding species could not be characterized in detail but are likely responsible for the additional COOH

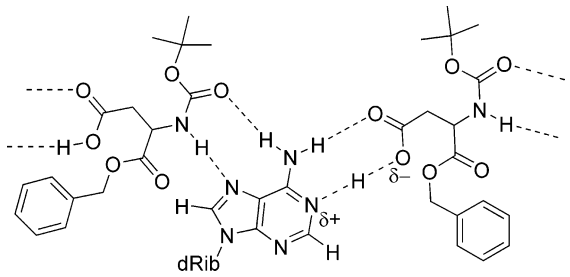

Figure 8. Potential higher order complexes between Boc-Asp-OBzl and adenosine.

resonances observed in the low-field region of corresponding 1D spectra at low temperatures (see Figures 2a and 6). Because of their multiple functionalities and favored by the low temperatures, it is even conceivable that the N-protected aspartic acid and the adenine nucleoside associate to form even more extended linear aggregates as indicated in Figure 8.

The broad resonances as seen in particular for mixtures of 1-^{15}N-rA and Ac-Asp-OMe at 128 K (Figure 6) may be associated with such extended complexes and/or exchange processes in weak adenine−adenine interactions that were also noticed in adenosine−acetic acid mixtures.[8]

Conclusions

The present studies show the formation of a strong hydrogen bond between the side chain carboxylic acid function of aspartate (pK_a = 3.9) and the N1 endocylic nitrogen of the adenine base (pK_a = 3.5). With matched pK_a's of proton donor and acceptor, the highly deshielded OH proton is found to be close on average to the center of the hydrogen bridge. However, hydrogen bond preferences are easily overridden by additional noncovalent interactions, hampering any prediction on the binding mode in case of more complex molecular systems and emphasizing the interplay of all forces involved. Thus, noncovalent interactions of appropriately positioned substituents on the adenosine and aspartic acid ligand shift complex formation toward a Hoogsteen configuration with a weaker OH···N7 hydrogen bond. Although introduced as protecting groups, the influence of the various bulky substituents in aspartic acid−adenosine complexation may lead the way for the future design of site-specific adenosine ligands.

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(14) ^{1}H NMR chemical shift for some protons in the major complex formed between 7-^{15}N-dA and Boc-Asp-OBzl: δ_H (500 MHz, Freon, 123 K) 6.11 (H1′), 7.81 (H2), 7.93 (H8), 8.14/9.99 (NH_2), 4.57 (Asp CH_α), 9.06 (Asp NH), 19.95 (Asp COOH).

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(16) ^{1}H NMR chemical shift for some protons in the major complex formed between 1-^{15}N-rA and Boc-Asp-OBzl: δ_H (500 MHz, Freon, 133 K) 6.15 (H1′), 8.41 (H2), 8.64 (H8), 7.38/8.20 (NH_2), 4.34 (Asp CH_α), 9.93 (Asp NH), 16.42 (Asp COOH).

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(18) Scalar coupling constants determined from 1D ^{1}H NMR spectra: J_{HH} (1:1 7-^{15}N-dA:Boc-Asp-OBzl, Freon, 273 K) 6.3 (H1′H2′), 6.3 (H1′H2″), 5.6 (H2′H3′), 4.1 (H2″H3′), 3.3 (H3′H4′), 4.3/4.5 (Asp CH_α-CH_β), 9.3 (Asp CH_αNH); J_{HH} (1:1 1-^{15}N-rA:Boc-Asp-OBzl, Freon, 273 K) 5.6 (H1′H2′), 5.6 (H2′H3′), 3.3 (H3′H4′), 4.1/4.5 (Asp $CH_\alpha CH_\beta$), 8.9 (Asp CH_αNH).